

**TRADE SECRET**

*Study Title*

H-28072: Static, Acute, 48-Hour Limit Test with *Daphnia magna*

**TEST GUIDELINES:** OECD Guideline for the Testing of Chemicals  
Section 2 (Part 202) (2004)

**AUTHOR:** Barbra D. Ferrell, B.S.

**ORIGINAL REPORT**

**COMPLETED ON:** December 6, 2007

**REPORT REVISION 1**

**COMPLETED ON:** July 14, 2008

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**WORK REQUEST NUMBER:** 17199

**SERVICE CODE NUMBER:** 241

**SPONSOR:** E.I. du Pont de Nemours and Company  
Wilmington, Delaware 19898  
U.S.A.

### GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are compatible with current OECD and MAFF (Japan) Good Laboratory Practices.

Study Director: Barbra D. Ferrell 14 July 2008  
Barbra D. Ferrell, B.S. Date  
Associate Scientist

## QUALITY ASSURANCE STATEMENT

Work Request Number: 17199  
Service Code Number: 241

Key inspections for DuPont work request 17199, service code 241 were performed for the tasks completed at DuPont by the Quality Assurance Unit of DuPont and the findings were submitted on the following dates.

<i>Phase Audited</i>	<i>Audit Dates</i>	<i>Date Reported to Study Director</i>	<i>Date Reported to Management</i>
Protocol:	June 1, 2007	June 1, 2007	June 1, 2007
Conduct:	June 5, 2007	June 5, 2007	June 5, 2007
Report/Records:	November 30, 2007	December 3, 2007	December 4, 2007
Report Revision 1:	July 8, 2008	July 8, 2008	July 8, 2008

Reported by: Donna M. Johnston 11 July 2008  
Donna M. Johnston Date  
Quality Assurance Auditor

### CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Reviewed by: Robert A. Hoke 9 July 2008  
Robert A. Hoke, Ph.D. Date  
Principal Research Ecotoxicologist and Manager

Issued by Study Director: Barbra D. Ferrell 14 July 2008  
Barbra D. Ferrell, B.S. Date  
Associate Scientist

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## STUDY INFORMATION

Substance Tested:

- HFPO Dimer Acid Ammonium Salt
- 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propionic acid, ammonium salt
- 62037-80-3 (CAS Number)
- H-28072

Haskell Number: 28072

Composition:

82.6%	Ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propionate*
13.9%	Water
3.5%	Ammonium
0.41%	Organic Impurities

\* Note: The Ammonium-2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propionate component (HFPO Dimer ammonium salt) contains 0.1 ppm HFPO trimer ammonium salt.

Purity: See composition, above

Physical Characteristics: Clear and colorless concentrated aqueous solution

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Study Initiated/Completed: May 30, 2007 / (see report cover page)

Experimental Start/Termination: June 5, 2007 / June 7, 2007

## REASON FOR REVISION 1

Consistent reporting of endpoints across studies, based on guidance contained in OECD TG 201, 202 and 203.

## SUMMARY

The acute toxicity of H-28072 to unfed *Daphnia magna* neonates, less than 24 hours old at test start, was determined in an unaerated, static, acute, 48-hour limit test. The test was conducted in accordance with the OECD Guideline for the Testing of Chemicals: 202.

The study was conducted with a 120 mg/L nominal concentration of H-28072 and a dilution water control at a mean temperature of 20.4°C (range of 20.1-20.7°C). The nominal H-28072 limit test concentration was 120 mg/L and the mean, measured concentration was 102 mg/L. The mean, measured limit test concentration was 80-120% of the nominal limit test concentration for the study. Six replicates with five daphnids per replicate were used for the limit test concentration and four replicates with five daphnids per replicate were used for the dilution water control.

No immobility was seen at the nominal 120 mg/L (102 mg/L mean, measured) H-28072 limit test concentration at the end of the 48-hour limit test. No immobility or sublethal effects were seen in the dilution water control daphnids. The 48-hour EC<sub>50</sub>, based on the nominal H-28072 limit test concentration and immobility, was greater than 120 mg/L.

The results are summarized as follows:

Nominal concentration of H-28072, mg/L <sup>a</sup>	dilution water control and 120
Mean, measured concentration of H-28072, mg/L	ND <sup>b</sup> , and 102
48-hour EC <sub>50</sub> for H-28072, based on nominal concentration, mg/L	greater than 120

a Not adjusted for 82.6% purity by analysis during preparation.

b ND denotes none detected at or above the limit of detection of 0.0001 µg/L.



## INTRODUCTION

The objective of this study was to assess the acute toxicity of H-28072 to unfed *Daphnia magna* neonates, less than 24 hours old at test start, during an unaerated, static, acute, 48-hour limit test.

## MATERIALS AND METHODS

### A. Test Guidelines

The study design complied with the following test guidelines:

- OECD, Section 2 (Part 202): Effects on Biotic Systems, *Guideline for the Testing of Chemicals* (2004).

### B. Test Substance

The test substance, H-28072, was supplied by the sponsor. The test substance purity was 82.6% by analysis (Appendix A).

The solubility and stability of H-28072 in HLWW at a nominal concentration of 120 mg/L was demonstrated under the conditions of the test and is shown by the analytical recoveries.

### C. Test Solution Preparation

The limit test concentration was prepared by adding approximately 0.24 g of test material to DuPont Haskell Well Water (HLWW) in a 2L glass beaker (final volume 2L) and stirring for approximately 40 minutes. The limit test concentration was clear and colorless with no visible precipitate.

### D. Dilution Water

Dilution water originated from the DuPont Haskell well which is 378-feet deep and is cased and sealed to bedrock. The hardness of the well water is adjusted to approximately 100-140 mg/L as CaCO<sub>3</sub> by the flow-proportioned addition of CaCl<sub>2</sub>. The well water is then aerated, passed through a green sand filter to remove iron, and filtered through 50-, 10-, and 5-μm filters to remove particulates. The water is heated or chilled as appropriate and distributed through aged polyvinyl chloride piping. The dilution water is analyzed twice yearly for major anions and cations, metals, total organochlorine and organophosphate pesticides, and polychlorinated biphenyls (Table 1). The dilution water meets OECD<sup>(1)</sup> specifications.

### E. Test Organism Culture

*Daphnia magna* were reared at DuPont Haskell in 1000-mL Pyrex<sup>®</sup> beakers (10 per beaker at culture initiation) which contained 1000 mL of aerated, filtered well water held at 20 ± 2°C. Daphnids were fed on a daily basis with 2 mL/L of a yeast, cereal leaves and trout chow (YCT)

mixture and the green alga, *Pseudokirchneriella subcapitata*, at a rate of approximately 62,500 cells/mL of culture media. The YCT mixture was standardized to 1700-2100 mg/L solids. The combination of YCT and alga is equivalent to 0.1-0.2 mg total organic carbon per daphnid per day. Neonates used in this test were less than 24 hours old and were collected from the 6<sup>th</sup> and 7<sup>th</sup> brood of 21-day old parent daphnids. Sickness, injury, and abnormalities were not seen and ephippia were not being produced by the parent daphnids. No adult immobility was seen during the 48-hour pretest period. *Daphnia magna* were identified by labels on the culture beakers and test chambers.

## **F. Test Methods<sup>(1)</sup>**

One nominal concentration and a dilution water control were used in this study. The nominal limit test concentration of H-28072 (not adjusted for 82.6% purity during preparation) of 120 mg/L was chosen for the definitive limit test based on the results of a preliminary rangefinding study.

Pyrex<sup>®</sup> beakers (250-mL) containing 200 mL of test solution (approximately 6.5-cm test solution depth) were used as test chambers. Six replicate test chambers with five daphnids per replicate were used for the limit test concentration and four replicates with five daphnids per replicate were used for the dilution water control. The test chambers were covered with a glass plate during the test. Random numbers were used to assign the position of test concentrations in the waterbath.

*Daphnia magna* neonates, less than 24 hours old, were used in this study. Daphnids were assigned to the test chambers using random numbers. Addition of daphnids to test solutions was initiated about 38 minutes after mixing of the test solutions was completed. Observations of test organisms were made daily. The criterion for the effect (immobility) was a lack of reaction to application of a gentle stimulus. Daphnids were not fed during the test.

A recirculating waterbath was used to maintain mean temperature in the test chambers during the 48-hour test at approximately 20.4°C with a range of 20.1 to 20.7°C (Table 5). In addition, a continuously-recording thermometer was used to check for temperature variations in the waterbath. A photoperiod of 16 hours light (approximately 453-544 Lux) and 8 hours darkness was employed, which included 30 minutes of transitional light (approximately 19-28 Lux) preceding and following the 16-hour light interval.

Dissolved oxygen concentration, pH, and temperature were measured in two replicates of the control and limit test substance concentration. These measurements were taken before daphnids were added at test start and at test end. Total alkalinity, EDTA hardness, and conductivity of the water and limit test concentration were measured before daphnids were added at the beginning of the test. Test solutions were not aerated during the test and were disposed of in an appropriate manner at test end.

## **G. Sample Preparation and Chemical Analysis**

### **1. Sample Collection**

A full description of sample preparation and chemical analysis is presented in Appendix B.

Samples and back-up samples from test solutions containing H-28072 at a nominal concentration of 120 mg/L and the blank control were transported on ice from DuPont Haskell to the analytical laboratory for concentration verification on test days 0 and 2.

Concentrations of H-28072 in test solution samples were measured by high performance liquid chromatography with detection by mass spectrometry (LC/MS/MS).

## RESULTS AND DISCUSSION

### H. In-Life Data

#### 1. Definitive Study

The nominal limit test concentration (not adjusted for 82.6% purity during preparation) for the definitive study was 120 mg/L. A dilution water control was used in this study. Four replicate chambers were used for the dilution water control. Six chambers were used per limit test concentration with five daphnids in each chamber (20 daphnids per control and 30 per limit test concentration). The mean, measured concentration of H-28072 was 102 mg/L and was within 80-120% of the nominal limit test concentration. No H-28072 was detected in the dilution water control.

Dilution water quality was acceptable based on OECD<sup>(1)</sup> dilution water criteria. Based on the most recent semi-annual dilution water analysis (Table 1), contaminant concentrations were below concentrations that could be expected to affect the integrity of a study. All chemical and physical parameters for the definitive test (Tables 3 - 6) were within expected ranges. Total alkalinity, EDTA hardness, and conductivity of the dilution water control and limit test concentration at test start ranged from 51 to 52 mg/L as CaCO<sub>3</sub>, 127 to 131 mg/L as CaCO<sub>3</sub>, and 255 to 285 µmhos/cm, respectively. During the test, dissolved oxygen concentrations ranged from 8.5 to 8.8 mg/L, pH ranged from 7.4 to 8.0, and mean temperature was 20.4°C with a range of 20.1 to 20.7°C.

Data on daily immobility and sublethal effects are presented in Tables 7 and 8, respectively. No immobility or sublethal effects were seen in the dilution water or at the nominal 120 mg/L (102 mg/L mean, measured) H-28072 limit test concentration at the end of 48 hours. The 48-hour EC<sub>50</sub>, based on the nominal H-28072 limit test concentration and immobility, was greater than 120 mg/L.

## CONCLUSION

H-28072 was assessed for acute toxicity to unfed *Daphnia magna* neonates, less than 24 hours old, in an unaerated, static, acute, 48-hour limit test. The 48-hour EC<sub>50</sub>, based on the nominal limit test concentration of H-28072 and immobility, was greater than 120 mg/L.

## RECORDS AND SAMPLE STORAGE

Specimens (if applicable), raw data, the protocol, amendments (if any), and the final report will be retained at DuPont Haskell, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware.

## REFERENCES

1. Organisation for Economic Co-Operation and Development (OECD). Guideline for the Testing of Chemicals: 202, 13 April 2004.

## TABLES

Table 1  
Chemical Characteristics of DuPont Haskell Well Water<sup>a</sup>

Parameter	MDL <sup>b</sup>	Analytical Value	Parameter	MDL <sup>b</sup>	Analytical Value
BOD, mg/L	3.1	ND <sup>c</sup>	Lead, mg/L	0.0069	ND
COD, mg/L	2.6	ND	Magnesium, mg/L	0.0322	3.82
DOC, mg/L	1.0	ND	Manganese, mg/L	0.00036	0.007
TOC, mg/L	1.0	ND	MBAS/LAS, mg/L	0.035	ND
Total Kjeldahl N, mg/L	0.5	ND	Mercury, mg/L	0.000056	ND
Ammonia N, mg/L	0.03	ND	Nickel, mg/L	0.0056	ND
Turbidity, NTU	0.09	ND	Nitrite, mg/L	0.015	ND
Phenolics, mg/L	0.015	ND	Nitrate, mg/L	0.04	ND
Color, apparent Co/Pt <sup>d</sup>	5.0	ND	Ortho-phosphate, mg/L	0.01	ND
Solids			Potassium, mg/L	0.0503	3.94
total suspended, mg/L	1.5	ND	Selenium, mg/L	0.0005	ND
Aluminum, mg/L	0.0802	ND	Silver, mg/L	0.000023	ND
Antimony, mg/L	0.0097	ND	Sodium, mg/L	0.433	8.15
Arsenic, mg/L	0.00067	ND	Sulfate, mg/L	0.3	6.9
Beryllium, mg/L	0.00094	ND	Sulfide, mg/L	0.054	ND
Boron, mg/L	0.0094	0.0212 J <sup>e</sup>	Zinc, mg/L	0.0081	ND
Bromide, mg/L	0.4	ND	Ca/Mg	NA <sup>f</sup>	11.81
Cadmium, mg/L	0.000099	ND	Na/K	NA	2.07
Calcium, mg/L	0.104	45.1	Volatile priority		
Chloride, mg/L	4.0	66.5	pollutants, µg/L	0.5-40	ND
Chlorine, residual, mg/L	0.04	0.040 J	Acid extractable		
Chromium, mg/L	0.00026	0.00041 J	priority pollutants, µg/L	0.9-19	ND
Cobalt, mg/L	0.0021	ND	Base/neutral		
Copper, mg/L	0.0002	ND	priority pollutants, µg/L	0.9-19	ND
Cyanide, mg/L	0.005	ND	Pesticides/PCBs, µg/L	0.0019-0.28	ND
Iron, mg/L	0.0522	ND	Organophosphate		
Fluoride, mg/L	0.08	0.32	pesticides, µg/L	0.47-0.95	ND

<sup>a</sup> Sample analyses performed at Lancaster Laboratories, Lancaster, Pennsylvania, date of sample collection 11 May 2007 unless indicated otherwise.

<sup>b</sup> MDL = method detection limit, <sup>c</sup> ND indicates not detected at the MDL, <sup>d</sup> Units based on cobalt/platinum reference, <sup>e</sup> A "J" follows analytical values which were greater than the MDL but less than the limit of quantitation, <sup>f</sup> NA = not applicable.

Table 2  
Water Chemistry of the Dilution Water Control and Limit Test Concentration at Test Start

Nominal H-28072 Concentration (mg/L)	Total Alkalinity (mg/L as CaCO <sub>3</sub> )	EDTA Hardness (mg/L as CaCO <sub>3</sub> )	Conductivity (µmhos/cm)
Dilution Water Control	51	131	255
120	52	127	285

Table 3  
Dissolved Oxygen Concentration (mg/L)<sup>Ψ</sup> of H-28072 Test Solutions

Nominal H-28072 Concentration (mg/L)	0 Hours		48 Hours	
	A <sup>†</sup>	C <sup>†</sup>	A <sup>†</sup>	C <sup>†</sup>
Dilution Water Control	8.5	8.5	8.7	8.7
120	8.5	8.5	8.8	8.7

Ψ The theoretical dissolved oxygen concentration at 100% saturation is 9.1 mg/L at 20°C.

† Replicate test chambers contained 5 daphnids each (total 30 daphnids for the limit test concentration and 20 for the dilution water control) at test start.

Table 4  
pH of H-28072 Test Solutions

Nominal H-28072 Concentration (mg/L)	0 Hours		48 Hours	
	A <sup>†</sup>	C <sup>†</sup>	A <sup>†</sup>	C <sup>†</sup>
Dilution Water Control 120	7.4	7.4	8.0	7.9
	7.6	7.6	8.0	8.0

† Replicate test chambers contained 5 daphnids each (total 30 daphnids for the limit test concentration and 20 for the dilution water control) at test start.

Table 5  
Temperature (°C) of H-28072 Test Solutions

Nominal H-28072 Concentration (mg/L)	0 Hours		48 Hours	
	A <sup>†</sup>	C <sup>†</sup>	A <sup>†</sup>	C <sup>†</sup>
Dilution Water Control 120	20.5	20.5	20.2	20.2
	20.5	20.7	20.1	20.2

† Replicate test chambers contained 5 daphnids each (total 30 daphnids for the limit test concentration and 20 for the dilution water control) at test start.



Table 6

Immobility of *Daphnia magna* at 24 and 48 Hours in an Unaerated, Static, Acute, 48-Hour Limit Test with H-28072

Nominal H-28072 Concentration (mg/L)	Number Immobile / Number at Test Start											
	24 Hours						48 Hours					
	A <sup>†</sup>	B <sup>†</sup>	C <sup>†</sup>	D <sup>†</sup>	E <sup>†</sup>	F <sup>†</sup>	A <sup>†</sup>	B <sup>†</sup>	C <sup>†</sup>	D <sup>†</sup>	E <sup>†</sup>	F <sup>†</sup>
Dilution Water Control 120	0/5	0/5	0/5	0/5	-	-	0/5	0/5	0/5	0/5	-	-
	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

† Replicate test chambers contained 5 daphnids each (total 30 daphnids for the limit test concentration and 20 for the dilution water control) at test start.

- Not applicable

Table 7  
Sublethal Effects in *Daphnia magna* at 24 and 48 Hours in an Unaerated, Static, Acute 48-Hour Limit Test with H-28072

Nominal H-28072 Concentration (mg/L)	Number Affected / Number Alive											
	24 Hours						48 Hours					
	A <sup>†</sup>	B <sup>†</sup>	C <sup>†</sup>	D <sup>†</sup>	E <sup>†</sup>	F <sup>†</sup>	A <sup>†</sup>	B <sup>†</sup>	C <sup>†</sup>	D <sup>†</sup>	E <sup>†</sup>	F <sup>†</sup>
Dilution Water Control 120	0/5 0/5	1 <sup>a</sup> /5 0/5	0/5 0/5	0/5 0/5	- 0/5	- 0/5	0/5 0/5	0/5 0/5	0/5 0/5	0/5 0/5	- 0/5	- 0/5

† Replicate test chambers contained 5 daphnids each (total 30 daphnids for the limit test concentration and 20 for the dilution water control) at test start.

- Not applicable

#### OBSERVATION KEY

a Daphnid floating at surface

## **APPENDICES**

**Appendix A**  
**Certificate of Analysis**



E. I. du Pont de Nemours and Company  
Wilmington, DE 19898  
USA

### CERTIFICATE OF ANALYSIS

This Certificate of Analysis fulfills the requirement for characterization of a test substance prior to a study subject to GLP regulations. It documents the identity and content of the test substance. This work was conducted under EPA Good Laboratory Practice Standards (40 CFR 792).

Haskell Code Number	H-28072
Common Name	HFPO Dimer Acid Ammonium Salt
Purity Percent	82.6%
Other Components	Water – 13.9% Ammonium (excess) – 3.5%
Date of Analysis	July 19, 2007
Recommended reanalysis interval	1 year
Instructions for storage	NRT&H
Reference	DuPont-23285
Analysis performed at	E. I. DuPont de Nemours and Company DuPont Haskell Laboratories Newark, Delaware USA

Peter A. Bloxham, Ph.D.  
Analyst's Name

  
Analyst's signature

20-JUL-2007  
Date

**Appendix B**  
**Analytical Report**

Test Solutions Analyses:

H-28072: Static, Acute, 48-Hour Limit Test with *Daphnia magna*

**AUTHOR:** Karen M. L'Empereur, Ph.D.

**ANALYTICAL STUDY COMPLETED ON:** July 19, 2007

**REPORT REVISED ON:** August 17, 2007  
September 19, 2007

**PERFORMING LABORATORY:** Critical Path Services (CPS)  
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Quillen Bldg., Suite 1-I  
Wilmington DE 19810

**SPONSOR:** E.I. du Pont de Nemours and Company  
Wilmington, DE 19898

**CPS PROJECT NUMBER:** 07-CPS-022

**SPONSOR PROJECT NUMBER** DuPont-22895, Rev. 2

**WORK REQUEST NUMBER:** 17199

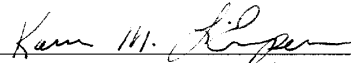
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### GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This analytical phase of this study was conducted in compliance with U.S. EPA TSCA (40 CFR Part 792) Good Laboratory Practice Standards, which are compatible with the OECD and MAFF Japan Good Laboratory Practice Standards.

CPS

Principal Investigator:

  
Karen M. L'Empereur, Ph.D.  
Critical Path Services

Date: 17-Sep-07

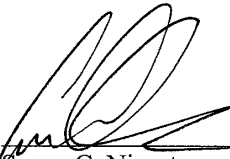


### QUALITY ASSURANCE STATEMENT

This study was inspected/audited by Quality Assurance according to CPS Standard Operating Procedures and EPA's Good Laboratory Practice Standards (40 CFR Part 792) and all findings were reported to the Study Director and Management. It was concluded that the final report accurately reflects the raw data for this study.

Phase Audited	Date of QAU Inspection	Date Reported to Study Director	Date Reported to Management
Sample Receipt	05-June-2007	05-June-2007	05-June-2007
Study Records, Final Report	17-July-2007	18-July-07	18-July-07
Revised Final Report	17-August-2007	17-August-2007	17-August-2007
Second Revision of Final Report	18-September-2007	18-September-2007	18-September-2007

CPS  
Quality Assurance Auditor:

  
Susan C. Nicastro  
Critical Path Services

Date: 18 Sept 2007

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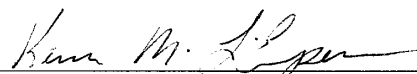
DuPont – 22895-AN, Rev. 2

### CERTIFICATION

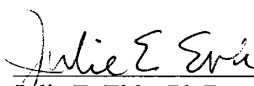
We, the undersigned, declare that this report provides an accurate evaluation of data obtained from the analytical phase of this study.

CPS

Principal Investigator:

 Date: 19-Sep-07  
Karen M. L'Empereur, Ph.D.  
Critical Path Services

CPS Management:

 Date: 19 Sept 2007  
Julie E. Eble, Ph.D.  
Critical Path Services  
Laboratory Director

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### SUMMARY

Samples from the limit test solutions containing H-28072 at a nominal concentration of 120 mg/L and the dilution water control were submitted for concentration verification on test day 0. Samples from 2 replicate chambers of the limit test concentration and the dilution water control were submitted at test end (day 2).

Concentrations of H-28072 in test solution samples were measured by high performance liquid chromatography with detection by mass spectrometry (LC/MS/MS).

The mean, measured value of H-28072 in the single limit test concentration was 103% of the targeted nominal test concentrations adjusted for test substance purity of 82.6%.

Control solutions contained no detectable concentrations of H-28072.

The report has been revised to reflect the change in the assigned purity of the H-28072 test substance from 81.4% to 82.6%. This change does not affect the conclusions of the study. Additionally, this report has been revised to correct a typographical error in Table 1 (the mean, measured concentration is 102 mg/L, not 103 mg/L).

## MATERIALS AND METHODS

### A. Sample Preparation and Chemical Analysis

#### 1. Sample Collection and Treatment

One sample plus a back-up sample of the test solution was received from the limit test concentration and the dilution water control on day 0 of the test before the test solutions were poured into replicate test chambers. One sample plus a back-up sample was also received from 2 replicate chambers (replicates B and D) of the limit test concentration and the dilution water control at test end (day 2). Samples and back-up samples were transported on ice to the analytical laboratory, and were stored refrigerated upon receipt and when not in use.

The samples including controls were diluted 2000x, with a solution of HPLC grade water/acetonitrile, 50/50, v/v, before analysis. Dilution of the samples was necessary due the sensitivity of the detector to H-28072.

Concentrations of H-28072 were measured by high performance liquid chromatography with detection by mass spectrometry (LC/MS/MS) in samples that were stored refrigerated and analyzed within 1 day of sample receipt.

## 2. Instrument and Conditions

HPLC Instrument: Agilent Model 1200  
MS Instrument: Applied Biosystems API 4000  
Software: Analyst 1.4.1

### LC Parameters:

Column: Phenomenex Luna C8; 150 x 2.0 mm, 5.0  $\mu$ m  
Mobile Phase: Premix of water/acetonitrile, 50/50, v/v, with 0.15% acetic acid and 0.15% triethylamine  
Flow Rate: 0.400 mL/min  
Column Temperature: 30  $^{\circ}$ C  
Injection Volume: 3.0  $\mu$ L

### MS Parameters:

Polarity (+/-)	Q1 (m/z)	Q2 (m/z)	Dwell (msecs)	CUR (psi)	GS1 (psi)	GS2 (psi)	TEM ( $^{\circ}$ C)	iHe	IS (V)	DP (V)	EP (V)	CE (V)	CXP (V)
-	329.00	285.00	100	40	11	60	400	on	-4500	-20	-10	-10	-5

## 3. Quantitation

A primary stock solution of the test compound, H-28072 (purity 82.6%), was made by dissolving the standard in water. Appropriate aliquots of the primary stock solution were diluted with dilution water (HLWW) to prepare a secondary stock solution. On each day of analysis the secondary stock solution was diluted with a solution of water/acetonitrile, 50/50, v/v to give calibration standards with concentrations that bracketed the concentrations of the diluted test solutions. Duplicate injections of test and calibration standard solutions were made and peak areas were determined electronically.

The calibration standard curve was generated by regression analysis using the chromatographic peak areas of the calibration standard solutions. Data for test solutions were compared to the calibration standard curve to determine concentrations of H-28072. The limit of detection (LOD) was determined by calculating the average noise level in chromatograms of the dilution water control solutions and comparing them to the signal of a calibration standard of known concentration. Two chromatograms were examined for noise-related peaks near the retention time of the analyte. The LOD was calculated as 3 times the concentration equivalent of the mean noise level. The limit of quantitation (LOQ) was defined as the greater of 10 times concentration equivalent of the mean noise level or the lowest standard concentration.

## RESULTS AND DISCUSSION

### A. Analytical Report

#### 1. Chromatographic Results

H-28072 eluted as a well-resolved chromatographic peak with a retention time of approximately 2.1 minutes. A typical calibration standard curve is shown in Figure 1. Representative chromatograms of a calibration standard solution, a dilution water control solution sample, and a test solution sample are presented in Figures 2 to 4, respectively.

The LOD and LOQ were determined to be 0.0001 µg/L and 25.9 µg/L, respectively.

#### 2. Test Solution Results

The mean, measured value of H-28072 in the limit test concentration was 103% of the targeted nominal test concentration adjusted for test substance purity of 82.6% (Table 1). All measured values of H-28072 were within 1.5X of the lowest value for all samples within the concentration. These data indicate that the H-28072 limit test concentration was maintained at acceptable levels throughout the definitive test.

Control solutions contained no detectable concentrations of H-28072 (Table 1).

TABLE 1  
MEASURED CONCENTRATIONS OF H-28072 IN TEST SOLUTIONS

Nominal H-28072 Concentration (mg/L)	Nominal H-28072 Concentration, Adjusted for Purity (mg/L) <sup>a</sup>	Measured H-28072 Concentration (mg/L)		Mean, Measured Concentration (mg/L) <sup>d</sup>	Percent Recovery (%) <sup>e</sup>
		Day 0 <sup>b</sup>	Day 2 <sup>c</sup>		
Water Control B <sup>f</sup>	0.0	ND <sup>g</sup>	ND	---	---
Water Control D <sup>f</sup>	0.0		ND		
120 B	99.1	103	102	102	103
120 D	99.1		100		
Average			101		

a Nominal H-28072 concentrations adjusted for 82.6% purity.

b One sample of freshly prepared test solution was taken from the test concentration solution and control solution on day 0 before they were poured into the replicate test chambers.

c Two replicates were sampled on day 2.

d Mean, measured H-28072 concentration was calculated as [Day 0 +(Day 2 Average)]/2.

e Based on nominal concentration adjusted for purity.

f Replicate identification.

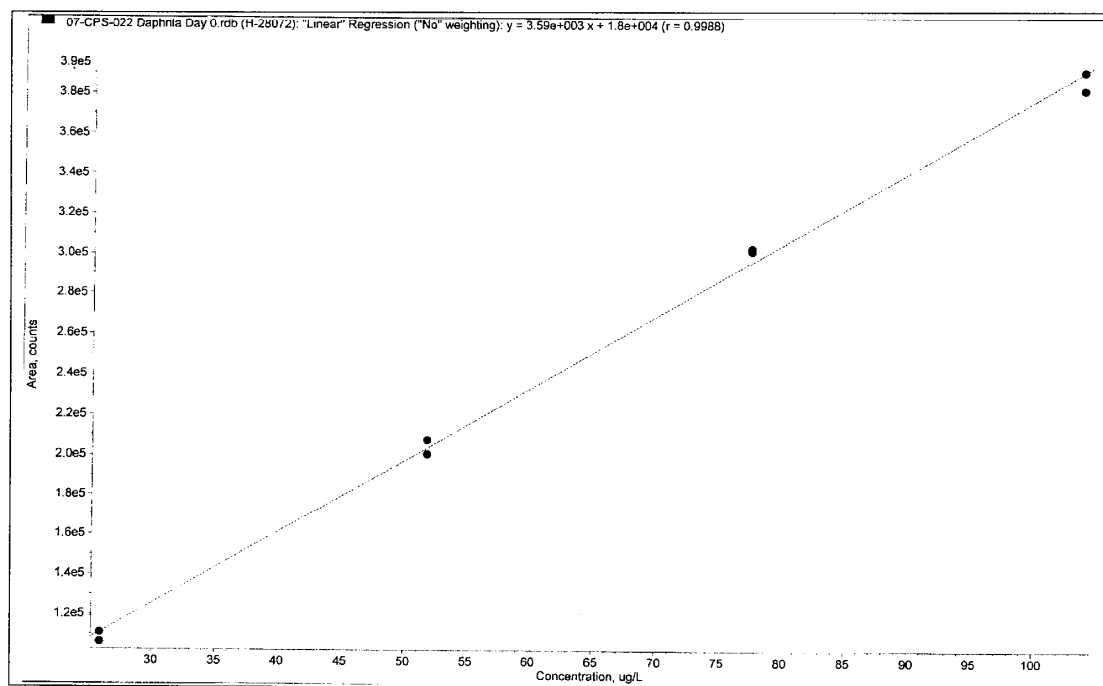
g ND denotes not detected. The limit of detection for H-28072 was calculated as 0.0001 µg/L.



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FIGURE 1

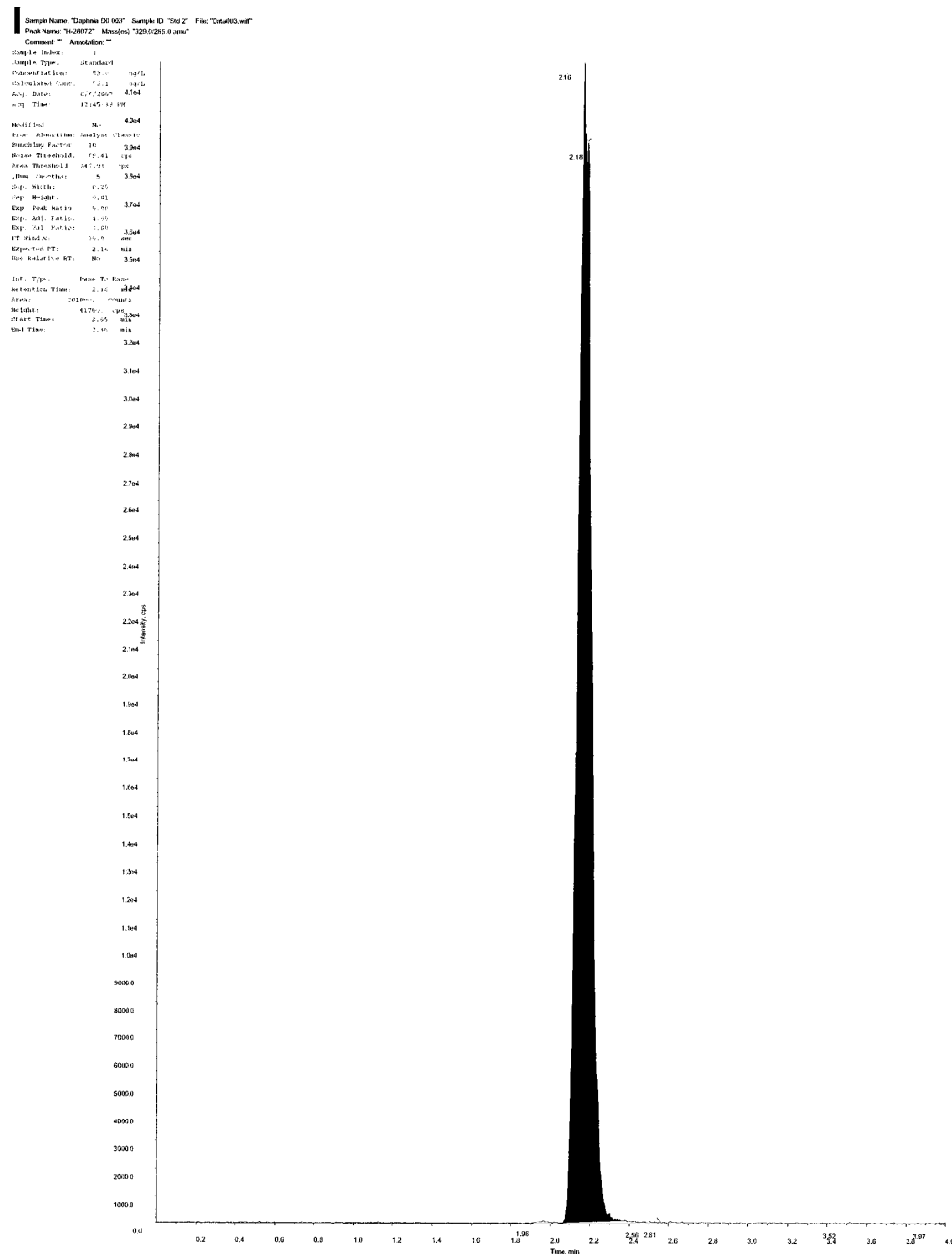
REPRESENTATIVE ANALYTICAL CALIBRATION STANDARD CURVE FOR H-28072



Concentration of H-28072 in  $\mu\text{g/L}$

FIGURE 2

### REPRESENTATIVE CHROMATOGRAM OF A CALIBRATION STANDARD SOLUTION

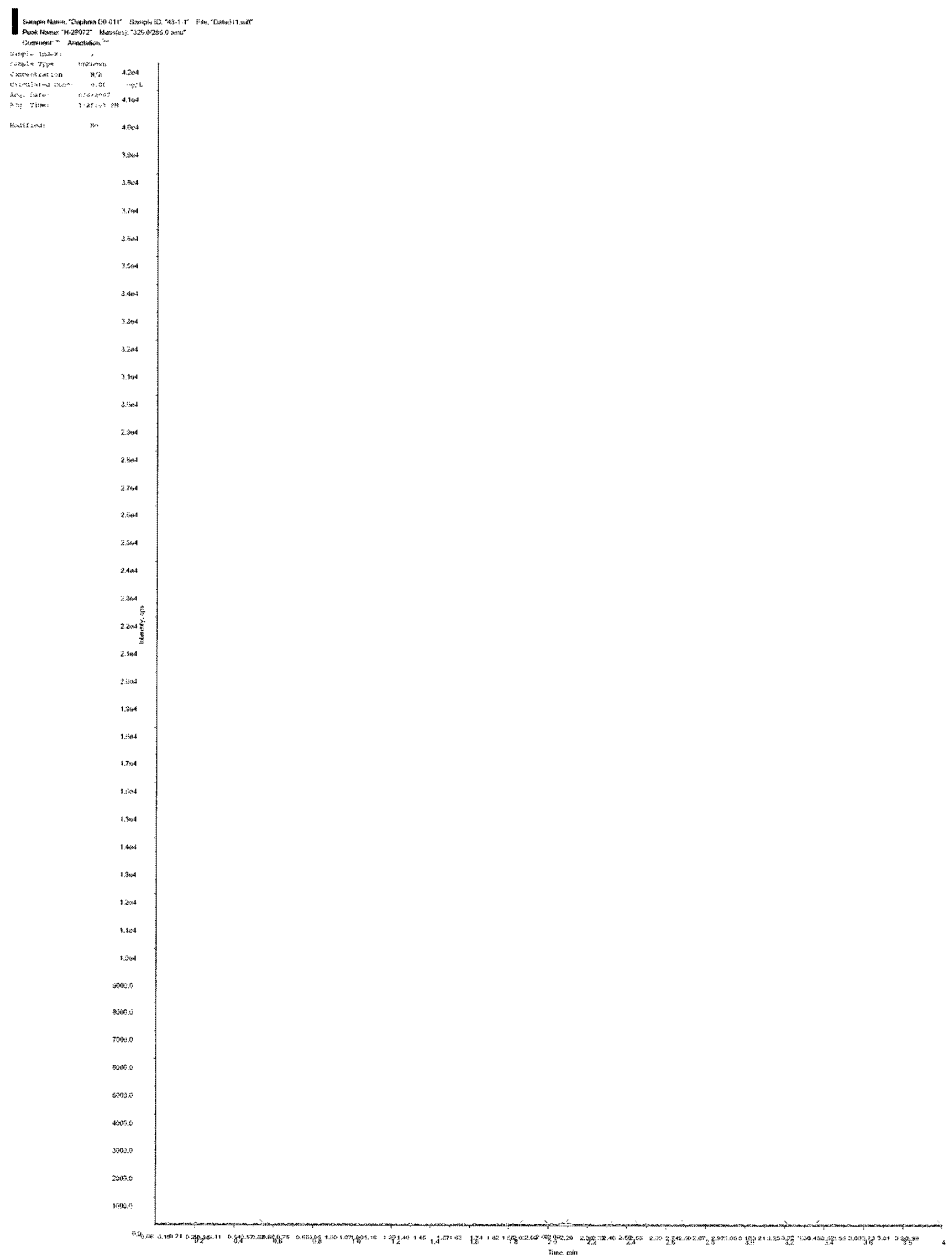


H-28072 elutes at a retention time of approximately 2.16 minutes. The calibration standard solution contains H-28072 at a concentration of 51.8 µg/L.

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FIGURE 3

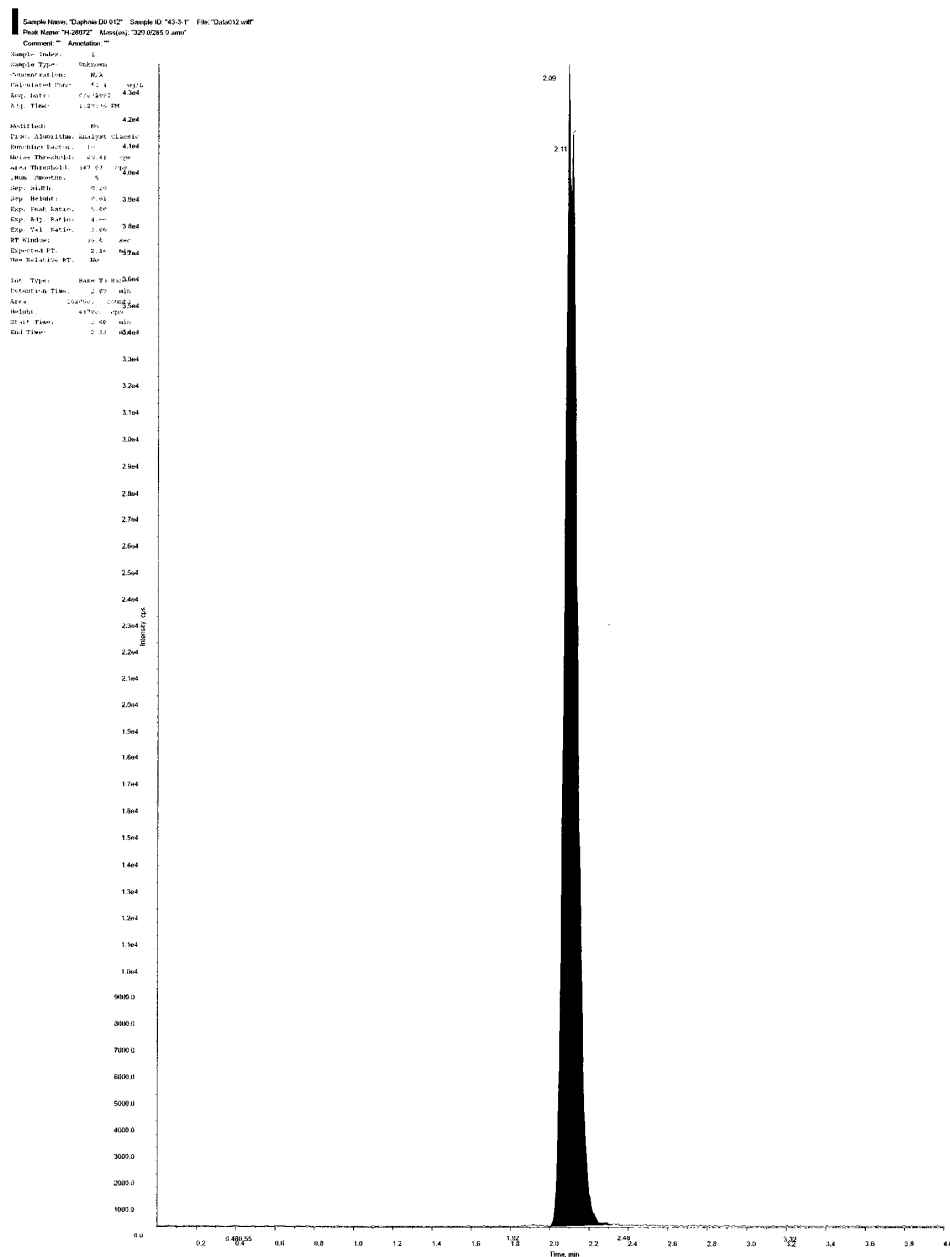
REPRESENTATIVE CHROMATOGRAM OF A DILUTION WATER CONTROL SOLUTION



H-28072 would elute at a retention time of approximately 2.16 minutes.

FIGURE 4

### REPRESENTATIVE CHROMATOGRAM OF A H-28072 TEST SOLUTION



H-28072 elutes at a retention time of approximately 2.11 minutes. The test solution sample contains H-28072 at a nominal concentration of 60 µg/L. The sample was diluted 2000x with acetonitrile/water, 50/50, v/v, prior to analysis.